TrkB signaling underlies the rapid antidepressant effects of isoflurane

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Neuronal plasticity induced by signaling through BDNF receptor TrkB has been implicated in the actions of antidepressants, including the rapid-acting antidepressant ketamine. We show that isoflurane induced transphosphorylation of TrkB by Src family kinases, stimulates the mTor signaling pathway and promotes neuronal plasticity and antidepressant-like behavior in rodents. Our findings provide a neurobiological basis for the clinically observed antidepressant effects of isoflurane and encourage its further evaluation as a rapid-acting antidepressant treatment devoid of the psychotomimetic side effects of ketamine.

Isoflurane anesthesia has been shown to produce rapid antidepressant effects in treatment-resistant depressive patients[1–4] (however, see[3,5,6]), but the unknown neurobiological mechanism has reduced the interest to further evaluate this treatment as an alternative for electroconvulsive therapy (ECT) and ketamine. We investigated the effects of isoflurane in the rat learned helplessness model with a good face, construct and predictive validity regarding depression[7,8]. A single isoflurane anesthesia produced an antidepressant-like effect (Fig. 1A), which is similar to that rapidly seen after ketamine[9], but requiring repeated treatment with classical antidepressants[8]. We also tested the antidepressant-like effects of isoflurane in an animal model of depression induced by persistent neuropathic pain[10]. Mice subjected to the sciatic nerve cuffing showed the expected depression-like phenotype in the Novelty-Suppressed feeding test (Fig. 1B). Remarkably, a single exposure to isoflurane anesthesia reversed this phenotype without affecting mechanical allodynia (Fig. 1B; Supplementary Fig. 1). Notably, classical antidepressants require weeks of administration to show similar effects[11].

Since BDNF (brain-derived neurotrophic factor) receptor TrkB has been proposed as a common target of antidepressants[12–15], we examined whether isoflurane might regulate TrkB signaling in rodents. Indeed, we found that a brief isoflurane anesthesia induces TrkB phosphorylation (pTrkB) in the medial prefrontal cortex (mPFC), hippocampus (HC) and somatosensory cortex (SCX) (Fig. 2A-B, Supplementary Fig. 2-5). Downstream of TrkB, isoflurane induced the phosphorylation of CREB (cAMP response element binding protein), Akt and GSK3β (glycogen synthase 3β), as well as mTOR (mammalian target of rapamycin) and its downstream kinases p70S6K and 4-EBP1 (Fig. 2C-H; Supplementary Fig. 2). Notably, these signaling effects are very similar to those induced by subanesthetic doses of ketamine[16,17]. Essentially similar changes in pTrkB were also observed after halothane and sevoflurane anesthesia (Supplementary Fig. 6).

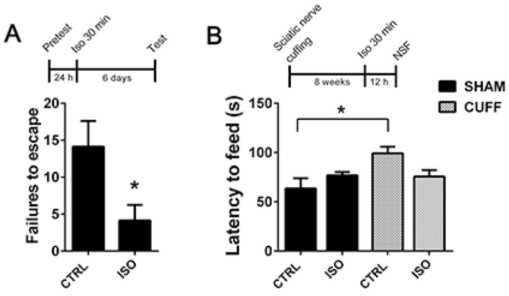

Fig. 1: Isoflurane produces antidepressant-like behavioural effects in rodents. (A) Single isoflurane anesthesia (30 min) decreases the escape failures in the learned helplessness test when tested 6 days after the anesthesia (p=0.0296, Student's t test; n=7). (B) Mice subjected to right common sciatic nerve cuffing show anxiodepressive behavior (increased latency to feed) in the Novelty-Suppressed Feeding test. Such phenotype is not seen in mice treated with isoflurane at 12 hours before testing (two-way ANOVA: cuffing*treatment interaction $F_{3,27}$=6,398, p=0.018, two-way ANOVA; $n_{sham\ ctrl}$=8, $n_{sham\ iso}$=8, $n_{cuff\ ctrl}$=8, $n_{cuff\ iso}$=7). (C) *p<0.05, **p<0.01. Abbreviations: CTRL, control treatment; ISO, isoflurane treatment; Sham, sham surgery; Cuff, cuff surgery (neuropathic pain).

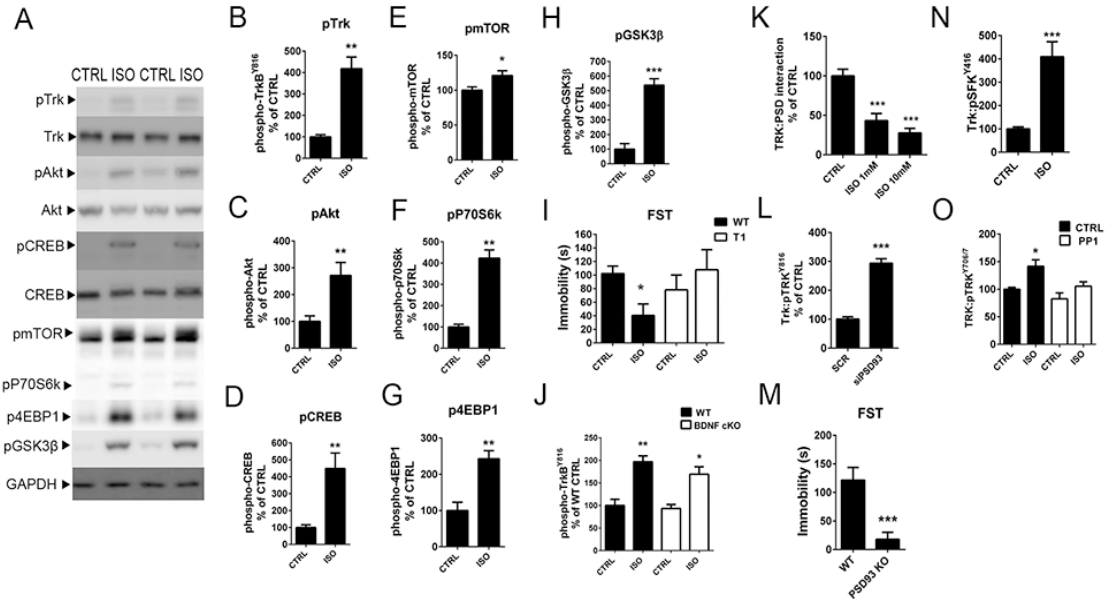

Fig. 2: Isoflurane induces TrkB transphosphorylation and signaling. (A) Representative western blots showing the effects of isoflurane anesthesia (30 min) on the phosphorylation of (B) TrkB (p=0.0022), (C) Akt^{T308} (p=0.0084), (D) CREB^{S133} (p=0.0087), (E) mTOR (p=0.0292), (F) p70S6K (p=0.0022), (G) 4-EBP1$^{T37/46}$ (p=0.0012) and (H) GSK3β^{S9} (p=0.0001) in the adult mouse prefrontal cortex. TrkB phosphorylation levels have been normalized to total TrkB, pAKT levels to total Akt, pCREB levels to total CREB, whereas other phosphoproteins to GAPDH. n=6/group. (I) Wild-type mice treated with isoflurane for 30 min show an increased latency to the first immobility when tested 15 minutes after the end of the treatment, whereas in the mice over-expressing the dominant-negative TrkB.T1 isoform the effect was absent. (two-way ANOVA genotype*treatment interaction $F_{3,24}$=4,301, p=0.049, n=7). (J) Isoflurane activates TrkB also in the hippocampus of BDNF cKO mice suggesting a transphosphorylation mechanism (Two-way ANOVA treatment effect $F_{3,12}$=41,843, p<0.001; Tukey HSD post hoc test WT CTRL vs. WT ISO p=0.001, WT CTRL vs. cKO ISO p = 0.015). (K) TrkB is bound to a complex with PSD-93 (CTRL) and isoflurane treatment dose-dependently reduces this interaction in RN33 cell homogenates *in vitro* (One-way ANOVA $F_{(2,12)}$=23,5, p<0.001. Tukey HSD post hoc test CTRL vs. ISO 1mM p=0.001; CTRL vs. ISO 10mM p<0.001). n=5/group. (L) Silencing PSD93 in RN33 cells results in increased phosphorylation of TrkB (p<0.001). (M) PSD-93 knockout mice show significantly reduced immobility time in the FST indicating an antidepressant-like phenotype (p<0.001). Female PSD93 KO mice were used in the experiment. (N) Isoflurane increased interaction between TrkB and activated Src in RN33 cells (p<0.001). (O) Pretreatment with PP1 (Src blocker) abolished the isoflurane induced TrkB phosphorylation in RN33 cells (Two way ANOVA followed by Tukey HSD post hoc test CTRL CTRL vs CTRL ISO p=0.02). *p<0.05, **p<0.01, ***p<0.001; Mann Whitney U test (B,D,F), Student's t test (C,E,G,H,J) or one-way ANOVA (K) or two-way ANOVA (I,J,O) followed by Tukey HSD *post hoc* test. Abbreviations: CTRL, control treatment; ISO, isoflurane treatment; CREB, cAMP response element binding protein; Akt, protein kinase B; mTOR, mammalian target of rapamycin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, GSK3β, glycogen synthase kinase 3β; SRC, scrambled. WT, wild-type; T1, mice overexpressing TrkB.T1.

TrkB activation has been shown to be necessary for the behavioral effects of antidepressants in the forced swim test (FST)[12–15]. Indeed, isoflurane produced rapid antidepressant-like behavioral responses in the FST[8] (Fig. 2I). These effects were likely not due to an increase in motor behavior, since isoflurane-treated mice showed reduced, rather than enhanced, locomotor activity (Supplementary Fig. 7). The effects of isoflurane in the FST were absent in mice over-expressing the dominant-negative TrkB.T1 isoform (Fig. 2I), suggesting that TrkB signaling is necessary for the effects of isoflurane in the FST.

Facilitation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor signaling and translation of BDNF through inhibition of eEF2 are suggested to govern the effects of ketamine on TrkB[9,12,16,18], yet we found no clear evidence supporting these mechanisms for isoflurane (Supplementary Fig. 8-9). BDNF levels were not altered at the time of TrkB phosphorylation, which occurs rapidly after isoflurane administration (Supplementary Fig. 10-11), but were increased later, at 1.5 h post-treatment (Supplementary Fig. 12). Most importantly, isoflurane readily activates TrkB in BDNF deficient mice (Fig. 2J), which indicates that BDNF is not required for acute TrkB activation, but may contribute to long-term effects of isoflurane.

To look for alternative mechanisms underlying the acute effects of isoflurane on TrkB, we immunoprecipitated TrkB interacting proteins from the mouse brain and identified them by quantitative mass spectrometry. Among the TrkB interacting proteins was PSD-93 (Supplementary Table 1), a synaptic scaffolding protein that is rapidly released from a protein complex with glutamate receptor subunits by volatile anesthetics in *in vitro* models[19] (Supplementary Fig. 13). We found that isoflurane dose-dependently disrupts PSD-93 - TrkB interaction in cell homogenates (Fig. 2K). Down-regulation of PSD-93 through siRNA resulted in increased pTrkB in cultured cells (Fig. 2L, Supplementary Fig. 14) indicating that the release from this protein complex allows TrkB activation. Consistently, we found that PSD-93 deficient mice showed prominent antidepressant-like behavior, i.e., reduced immobility in the FST although they showed decreased locomotor activity (Fig. 2M; Supplementary Fig. 15). Isoflurane also disrupted an interaction between PSD-93 and the Src family kinase Fyn that is known to transphosphorylate TrkB[20,21] and PSD93[22] (Supplementary Fig. 14D). Isoflurane promoted the interaction between TrkB and activated form of Fyn (Fig. 2N, Supplementary Fig. 14E), and the Src/Fyn inhibitor PP1 abolished isoflurane-induced pTrkB *in vitro* (Fig. 2O). Collectively these data suggests that isoflurane disrupts TrkB-PSD-93 protein complex and thereby promotes TrkB transphosphorylation through Src family kinases[21].

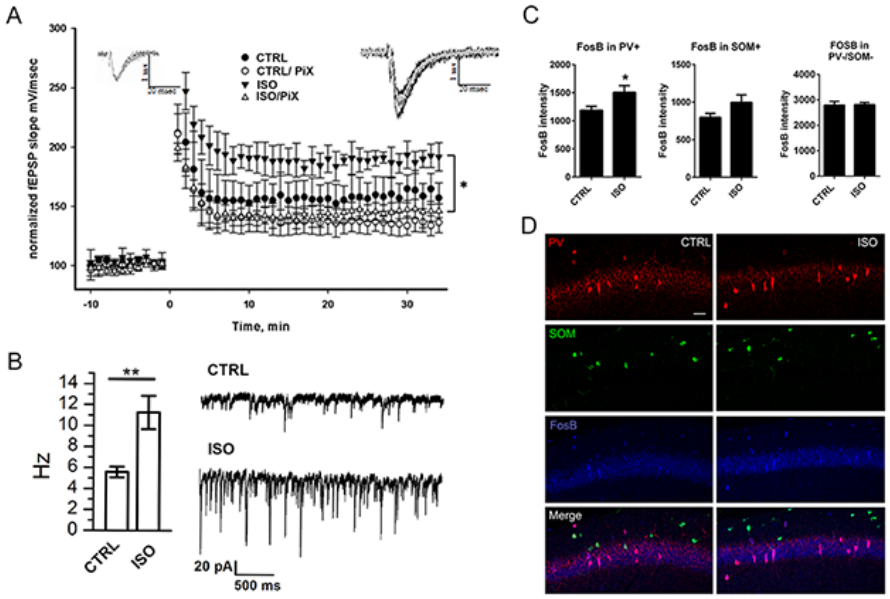

Fig. 3: Isoflurane accentuates synaptic function in the hippocampus. (A Long-term potentiation (LTP) induced by high-frequency stimulation (HFS, 100 Hz) is significantly enhanced in slices from mice treated with isoflurane for 30 min 24 hours before. The difference between the groups disappears in the presence of picrotoxin (PiX). Representative fEPSPs taken 5 min before and 30 min after the HFS are shown in the insets (control=black, isoflurane=dark grey; control/PiX=light gray; isoflurane/PiX=gray). (B) The average frequency of spontaneous IPSC in CA1 hippocampal neurons recorded at 24 hours after isoflurane anesthesia (30 min) (6 slices/6 animals per group, ANOVA, **p<0.01) and example traces of the recording. (C) The intensity of FosB staining in parvalbumin positive (PV+) cells, but not somatostatin positive cells (SOM+) or cells not expressing PV or SOM (PV-/SOM-), is significantly increased in the CA1 area of hippocampus of mice treated with isoflurane 24 hours before (p=0.0474, Student's t test). (D) Representative figures of the parvalbumin, somatostatin and FosB stainings. *<0.05, **<0.01.

Ketamine has been shown to promote synaptogenesis in the PFC of rats through the BDNF-TrkB-mTOR pathway[16,23]. However, in fixed sections of the PFC, HC or the SCX from mice anesthetized with isoflurane 24 hours before, spine density and morphology were not significantly different from those in the control mice (Supplementary Fig. 16). We further used in vivo 2-photon time-lapse microscopy to image dynamics of dendritic spines in the SCX in the same awake mouse (head-fixed but otherwise freely moving in the Mobile HomeCage[24]) at 24 hours before (control condition), immediately before, and at 24 hours after a brief isoflurane anesthesia. Isoflurane had no effects on the spine formation and elimination rates, indicating that neither spine density nor spine dynamics were significantly affected by isoflurane anesthesia in this region (Supplementary Fig. 17). Next, we used electrophysiology to study the effects of isoflurane on synaptic plasticity[25]. Tetanic stimulation (100Hz/1s) of the Schaffer collateral - CA1 pathway in hippocampal slices prepared from mice treated with isoflurane 24 hours before produced a significantly higher long-term potentiation (LTP) of the field excitatory postsynaptic potentials (fEPSPs) than what was seen in the control slices (Fig. 3A, Supplementary Fig. 18B)[26]. Recordings of input–output relationship showed that isoflurane accentuated basal synaptic transmission in the HC (Supplementary Fig. 18A). Paired pulse facilitation was not affected by isoflurane suggesting unaltered release probability and presynaptic function (Supplementary Fig. 18C). These effects of isoflurane on synaptic strength resemble those of antidepressant fluoxetine, but appear much faster[11]. Picrotoxin abolished the accentuated LTP suggesting a $GABA_A$ dependent mechanism (Fig. 3A). To test this possibility we examined IPSCs (inhibitory postsynaptic currents) in CA1 pyramidal neurons and observed increased spontaneous GABAergic activity in slices obtained from isoflurane treated animals (Fig.3B; Supplementary Fig. 19A-C). These changes were associated with increased immunoreactivity of FosB, a marker of neuronal activity, specifically in parvalbumin interneurons in the HC and mPFC of isoflurane treated animals (Fig. 3C-D; Supplementary fig 20). Tetanic stimulation can easily result in PV-IN-driven GABAergic excitation and synchronous spiking in hippocampal pyramidal neurons thus promoting LTP induction[27,28] Altogether these studies suggest that a brief isoflurane anesthesia brings long-lasting changes on GABAergic excitability.

Our study demonstrates that isoflurane, using a dosing regimen shown to produce antidepressant effects in humans[1,3,4], activates the TrkB-mTOR signaling pathway that is implicated in antidepressant responses and produces long-lasting plasticity-related physiological and behavioral changes. These effects resemble those previously found with clinically used antidepressants, including ketamine[7,10,12,20]. However, while ketamine has been shown to increase glutamate release and AMPA receptor signaling, leading to TrkB activation through local BDNF translation and release, our data suggest that isoflurane induces a transphosphorylation of TrkB by Fyn kinase after the dissociation of a PSD-93 protein complex. This effect appears different from the dissociation of TrkB from PSD-95, which attenuates TrkB signaling[29] (Supplemental Fig 21). Moreover, isoflurane significantly potentiated synaptic function but did not affect spine morphology, while ketamine affects both the number and function of spines[16,18]. This is consistent with observations showing that isoflurane anesthesia regulates dendritic spine number and

morphology in the developing, but not in the adult brain[30]. These differences in the mechanisms of action of ketamine and isoflurane may become instrumental in the attempts to focus drug discovery efforts towards the shared pathways activated by diverse rapid-acting antidepressants. The long-lasting physiological (Fig. 3) and behavioral (Fig. 2) responses to a brief isoflurane anesthesia should be also taken into account when interpreting the data produced by experimental conditions involving isoflurane.

Collectively, our study provides a plausible neurobiological basis for the antidepressant effects of isoflurane anesthesia observed in some clinical trials and encourages further evaluation of isoflurane as a rapid-acting treatment against treatment-resistant depression devoid of cognitive side effects of ECT (Supplementary Fig. 22) and hallucinogenic properties of ketamine.

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Conflict of interests:
L.K. is a paid employee in Neurotar Ltd.

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